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Maintenance of imaginal disc plasticity and regenerative potential in *Drosophila* by p53

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ABSTRACT

Following irradiation (IR), the DNA damage response (DDR) activates p53, which triggers death of cells in which repair cannot be completed. Lost tissue is then replaced and re-patterned through regeneration. We have examined the role of p53 in co-regulation of the DDR and tissue regeneration following IR damage in *Drosophila*. We find that after IR, p53 is required for imaginal disc cells to repair DNA, and in its absence the damage marker, γ-H2AX is persistently expressed. p53 is also required for the compensatory proliferation and re-patterning of the damaged discs, and our results indicate that cell death is not required to trigger these processes. We identify an IR-induced delay in developmental patterning in wing discs that accompanies an animal-wide delay of the juvenile-adult transition, and demonstrate that both of these delays require p53. In *p53* mutants, the lack of developmental delays and of damage resolution leads to anueploidy and tissue defects, and ultimately to morphological abnormalities and adult inviability. We propose that p53 maintains plasticity of imaginal discs by co-regulating the maintenance of genome integrity and disc regeneration, and coordinating these processes with the physiology of the animal. These findings place p53 in a role as master coordinator of DNA and tissue repair following IR.

Introduction

The imaginal discs of *Drosophila*, larval organs that contain the adult progenitor cells, have remarkable regenerative properties such that loss of over half of disc cells through IR or other tissue damage can be replaced to reestablish an organ of relatively appropriate size (Bergantinos et al., 2010a; Bryant and Simpson, 1984; Haynie and Bryant, 1977; Haynie and Bryant, 1976). Damage of imaginal discs induces a continuum of processes with systemic and local components. At the site of damage local events occur including cell death, wound healing, cell cycle arrest, compensatory proliferation and re-patterning. At the same time a systemic developmental delay is activated that ensures that the replacement of damaged tissue is coordinated with the physiology of the animal as a whole. How the local and animal-wide events are regulated and coordinated in time is unknown.

Imaginal discs have long been a powerful experimental system for study of organ development and regeneration. Growth and patterning of the discs are tightly linked and occur primarily during the larval stage where they are fueled by feeding. Terminal differentiation occurs during the pupal stage after cell proliferation has ceased. At the onset of larval development disc cells are multipotent, and over time acquire cell fates in a process regulated by signaling activity from the disc pattern-organizers, Wingless and Dpp. disc regeneration is dependent upon the activity of the organizers, and can be induced by ectopic expression of Wg and Dpp (Johnston, 2005; Struhl and Basler, 1993; Sustar and Schubiger, 2005). The ability of imaginal discs to regenerate – their plasticity – requires that the cells remain in a state that is permissive to cell proliferation. By the end of the larval feeding period, the growth and patterning of discs are essentially complete and the larvae migrate away from the food to pupariate (PP). In the wing disc, two additional mitoses occur early in the pupal stage prior to metamorphosis (Hartenstein and Posakony, 1989; Schubiger and Palka, 1987). The onset of PP marks the end of the major period of disc proliferation and is controlled by a developmental clock that is regulated by the ring gland and other larval endocrine organs (Bodenstein, 1950). Damage to discs slows the larval developmental clock and the discs are repaired during an extended period of feeding and cell proliferation. Evidence has suggested that it is the continued proliferation of the discs that inhibits progression of the clock (Poodry and Woods, 1990; Simpson et al., 1980), although the mechanism by which this occurs is unknown.

Gamma-irradiation (IR) can cause loss of imaginal disc cells and lead to disc regeneration. In this case, regeneration is preceded by the DNA damage response (DDR), a highly conserved cellular response to DNA damage that is rapidly activated to repair genomic lesions. In the DDR, the DNA damage sensor Ataxia Telangiectasia mutated (ATM) recruits and phosphorylates repair proteins to the DNA break site (Niida and Nakanishi, 2006; Shiloh, 2003; Zhou et al., 2000). Among its targets is the histone H2A variant, H2AX, widely present in nucleosomes (Rogakou et al., 1998) but specifically phosphorylated (γ-H2AX) within minutes of DNA breakage, thus marking the sites of damage (Greer et al., 2003;

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Srivastava et al., 2009). *Drosophila* H2Av, the functional homolog of H2AX, is phosphorylated with similar kinetics and specificity after IR and is recognized by mammalian γ-H2AX antibodies (Leach et al., 2000; Madigan et al., 2002). The checkpoint kinase Chk2 is also an ATM target that, upon activation, phosphorylates and activates p53 (Brodsky et al., 2004; Chehab et al., 2000; Flaggs et al., 1997; Guo et al., 2000; Hirao et al., 2000). In *Drosophila*, *p53* mRNA expression is also significantly upregulated after DNA or tissue damage (Brodsky et al., 2000; Wells et al., 2006). In mammalian cells, p53 mediates a cell cycle arrest (Bunz et al., 1998; Kuerbitz et al., 1992), whereas in *Drosophila* the arrest is mediated by Chk1 and is p53-independent (Brodsky et al., 2000; Fogarty et al., 1997). The arrest in cell division facilitates DNA repair, but if repair is unsuccessful damaged cells are instructed to die by apoptosis. In the *Drosophila* DDR p53 plays a key role by regulating expression of the proteins that induce apoptosis, and also several DNA repair proteins (Brodsky et al., 2000; Brodsky et al., 2004; Sogame et al., 2003).

Previously, we found that *p53* is required for the regeneration response of imaginal disc cells damaged by co-expression of the apoptosis-inducing genes *reaper (rpr)* or *hid* and the caspase inhibitor P35 (hereafter referred to as *rpr*/*hid* (RH)-induced damage) (Wells et al., 2006). RH damage initiates cell-autonomous apoptosis yet prevents cell elimination and results in a regeneration response that is similar to that induced after IR or surgery. Several processes occur that are autonomous to the damaged tissue, including ectopic expression of Wingless, blastema formation, and compensatory proliferation (Haynie and Bryant, 1976; McClure et al., 2008; Smith-Bolton et al., 2009; Sustar and Schubiger, 2005; Wells et al., 2006). In addition, non-autonomous responses are induced: many non-damaged cells within the disc undergo apoptosis or slow cell division, and the larval developmental clock is slowed, delaying progression to the pupal stage (Sustar and Schubiger, 2005; Wells et al., 2006). p53 is activated upon RH damage and is required for both the autonomous and non-autonomous events (Wells et al., 2006), suggesting that p53 activity might not be restricted to the cells with RH-damage.

In addition to RH-induced tissue damage, blastema formation induced by ectopic expression of Wg requires p53, suggesting that p53 may have a general role in tissue repair (Wells et al., 2006). Moreover, as the DDR and tissue regeneration occur in strict sequence after IR, to promote survival of the organism after tissue damage a role for p53 in their temporal coordination seems likely. To investigate the generality of p53's requirement in regeneration and to determine the nature of the relationship between the DDR and tissue regeneration we studied the role of p53 in IR-induced regeneration and its coordination with the DDR.

We report here that p53 is also critical for disc regeneration following IR, suggesting that p53 universally promotes regeneration of damaged imaginal discs. Although initially the DDR occurs normally in *p53* mutant disc cells, we find that they are unable to repair the DNA lesions. In addition, lack of timely cell death in p53 mutants allows the damaged cells to persist in the epithelium, leading to numerous adult defects and reduced animal survival. However, we find that the absence of the early cell death program in the mutants cannot explain the necessity for p53 in regeneration. In addition, our results suggest that p53 controls a developmental patterning checkpoint in discs and the larval developmental timer. As a whole, our results suggest that p53 maintains plasticity of imaginal discs by integrating the maintenance of genome integrity with disc regeneration and coordinating these processes with the physiology of the animal.

Materials and methods

Fly strains

The following strains were used in this study. Unless otherwise indicated, strain descriptions can be found at http://flybase.bio.indiana.edu. All mutant lines were crossed into the background of the *yw* strain used as a control.

UAS-*P35* (Johnston, 2009)
UAS-*hid* (Huh et al., 2004)
$p53^{ns}$ (Sogame et al., 2003)
$p53^{5a\text{-}1\text{-}4}$ (Rong et al., 2002)
$chk2^{P6}$ (Brodsky et al., 2004)
EnGal4, UAS-*GFP* (Neufeld et al., 1998)
rpr150-lacZ (Brodsky et al., 2000)
wg-lacZ
*yw*hsflp^{122}TubGal4, *UAS-GFP*; FRT82B TubGal80 hs*CD2* (de la Cova et al., 2004)
*yw*hsflp^{122}; FRT82B *arm-lacZ*
FRT82B TubGal80 hsCD2 $p53^{ns}$ (gift of C. de la Cova)
UAS-*p53*
yw; TubGal80^{ts} (II)
HhGal4, UAS-*GFP*
$dronc^{I29}$/TM6B (Xu et al., 2005)
Act>*y*+>Gal4, UAS-*GFP*
*yw*hsflp^{122}; +; +
yw;+;+ (used as control strain).

Fly husbandry

Eggs from appropriate crosses were collected on yeasted grape plates for 2–3 h. After hatching, larvae were transferred to standard molasses food vials (≤50/vial to avoid crowding) supplemented with fresh yeast paste and raised at 25 °C for defined periods of time, as described (Johnston and Sanders, 2003; Neufeld et al., 1998).

Clonal induction for autonomous p53RE and ectopic Wg detection

ActGal4 flp-out clones were induced at 48 or 60 h AEL with an 8 min. heat shock at 37 °C. Animals were dissected between 100 and 120 h AEL.

p53 MARCM clone analysis

The $p53^{ns}$ allele was recombined onto the FRT82B chromosome and used in MARCM experiments (gift of C. de la Cova). For analysis of ectopic Wg expression clones were induced between 30 and 48 h AEL with a 20 min heat shock at 37 °C. Larvae were raised at 25 °C and dissected between 120 and 130 h AEL.

Gal80^{ts} experiments

Larvae were raised at 18 °C, which is permissive for Gal80 expression, for 144 h to allow growth of the animal without expression of Gal4-driven transgenes. Larvae were then shifted to the restrictive temperature (30 °C) to allow transgene expression for 40 h before dissection.

Irradiation

Larvae were given a dose of 40 Gy while in standard molasses food at specified times AEL using a Nordion gamma-cell 220 irradiation unit with a ^{60}Co source, and dissected at indicated times following IR.

Immunocytochemistry

Fixation and immunocytochemistry of imaginal discs were carried out as described (Johnston and Edgar, 1998). TUNEL assays were carried out using Apoptag Red (Intergen); a detailed protocol is available upon

request. Images were acquired using Apotome software and a Zeiss Axioplan 2 microscope with an Orca-100 CCD camera (Hammatsu) and processed with Photoshop (Adobe) software. The following antibodies and dilutions were used: mouse anti-CD2, 1:200; rabbit anti-H3P (Upstate), 1:1000; mouse anti-γ-H2AX (Upstate), 1:250; guinea pig anti-Sens (gift of H. Bellen), 1:1000; mouse anti-Delta (DSHB), 1:200; guinea pig anti-Kni (gift of J. Parker), 1:1000; mouse anti-Nrt (DSHB), 1:30; mouse anti-Wg (DSHB), 1:30; rabbit anti-ß-gal (Cappel), 1:2000; rabbit anti-Hid (gift of B. Hay), 1:1000; rabbit anti-cleaved Caspase 3 (Cell Signaling), 1:100; mouse anti-BrdU (Roche), 1:100; guinea pig anti-Dronc (Xu et al., 2005), 1:1000.

Results

p53 is required cell-autonomously in tissue regeneration and its expression is sufficient to induce a regeneration response in wing imaginal discs

After RH-damage, p53 is required for the regeneration response in the damaged disc and also for the systemic developmental delay that accompanies disc regeneration. However, *p53* mRNA expression is induced specifically in the damaged disc cells (Wells et al., 2006). To determine whether p53 activity was autonomous to the damaged tissue we took two approaches. First, we induced RH damage in wing discs and examined the extent of p53 activation with an activity reporter consisting of tandem p53 consensus binding sites in front of the lacZ gene (Brodsky et al., 2000). RH damage created in posterior cells by expressing UAS-*hid* and UAS-*P35* with Engrailed Gal4 induced p53 activity specifically in the damaged cells (data not shown) (Wells et al., 2006). Likewise, RH damage created in small 'flp-out' Gal4 clones of cells resulted in cell-autonomous activation of the p53 response element reporter (Fig. 1A). We also examined expression of the p53 activity reporter in other tissues in the larvae, including the fat body, ring gland, and Dilp-expressing neurons, but found no change in reporter activity in these tissues in response to RH damage (data not shown).

Second, we used the $p53^{ns}$ null allele (Sogame et al., 2003) in the MARCM system (Lee and Luo, 1999) to remove *p53* function specifically in RH-damaged cells and looked for ectopic expression of Wg, an early step in regeneration that is p53-dependent (Smith-Bolton et al., 2009; Wells et al., 2006). Of control MARCM clones in which RH damage was created with Hid + P35 expression, 74% (n = 87) showed robust and completely autonomous ectopic Wg expression in many cells within the clone (Fig. 1B"). The expression of a *wg-lacZ* reporter was identical to that of Wg protein (data not shown). In contrast, only 12% (n = 305) of *p53* mutant RH-expressing clones contained cells that expressed ectopic Wg (Fig. 1C–C"), and the expression was generally limited to one or two cells. Occasionally wild-type cells with Wg expression in ectopic locations can be found under normal physiological conditions (9% of cells, n = 91 discs), thus we infer that the cells expressing ectopic Wg in *p53* mutant RH-MARCM clones represented background. The endogenous pattern of Wg expression was unaffected by RH damage in *p53* mutant cells (Fig. 1C"). As p53 is activated cell-autonomously within RH damaged cells and is required cell-autonomously for the ectopic expression of Wg during regeneration, we conclude that p53 activity and function is restricted to the damaged cells.

The cell autonomous function of p53 in wing disc cells led us to ask whether p53 activation was sufficient to induce a regeneration

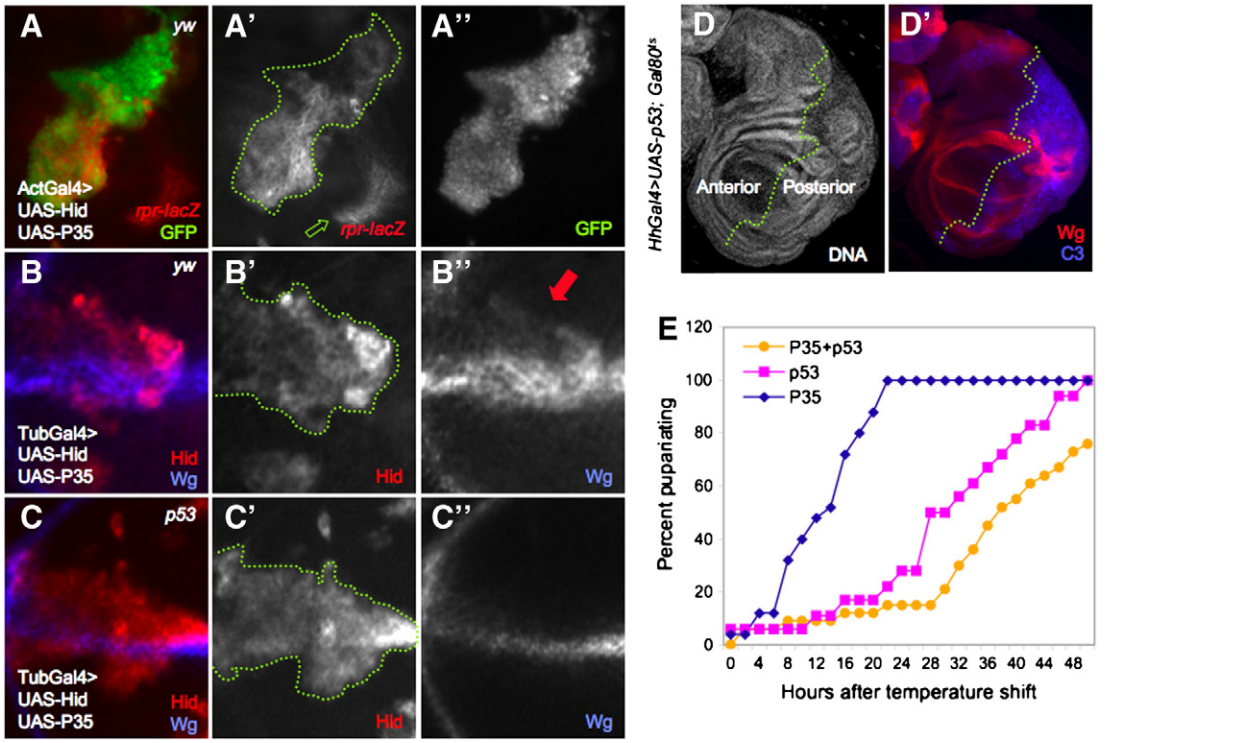

Fig. 1. *p53* activity is autonomous to damaged cells and is sufficient to induce a regeneration-like response in the wing disc. A. Act > y + > Gal4 'flp-out' clones expressing UAS-*Hid* + UAS-*P35*, marked by UAS-*GFP* expression (A"). The p53 activity reporter, $rpr^{150\text{-}lacZ}$, is autonomously activated within the boundaries of the clone (A', outlined in green). Green arrow indicates p53-independent reporter expression (see Supp. Fig. 1A–D). B–C. MARCM clones expressing UAS-*hid* + UAS-*P35* in the presence (B) or absence (C) of *p53*. In a *yw* background (control), ectopic Wg expression is autonomous to Hid + P35-expressing clones (Hid expression and dotted green line marks the clone, B'). Endogenous Wg expression (clones at the D/V boundary are shown in B and C) is also evident. All cells that express Wg ectopically are also Hid-positive (B", red arrow). C–C". Loss of *p53* specifically within UAS-*hid* + UAS-*P35*-expressing clones do not disturb endogenous expression of Wg but prevents its ectopic expression (Hid expression marks the clone). D. Expression of p53 for a limited period of time induces tissue repair (see text for details). HhGal4 > UAS-*p53* induces overgrowth and expression of ectopic Wg and C3 in posterior cells (D'). E. Developmental timing is delayed after transient induction of UAS-*p53* expression. Larvae expressed UAS-*P35* ± UAS-*p53* under HhGal4 control for 40 h (see text for details). The presence of $Gal80^{ts}$ in these animals did not affect growth or cell viability in the absence of UAS-*p53* (E). Expression of UAS-*p53* significantly delayed the time to pupariation relative to controls that expressed P35 alone. Co-expression of p53 and P35 exacerbated the delay. X-axis is expressed as hours after induction of Gal4 activity by Gal80 inactivation at 29 °C.

response in wing discs. Like expression of RH genes, expression of p53 from an early larval stage led to rapid cell death when in the absence of P35 (data not shown) (Huh et al., 2004; Ryoo et al., 2004; Wells et al., 2006). To circumvent this problem we expressed UAS-*p53* in posterior cells using Hedgehog (Hh) Gal4 under control of a temperature sensitive Gal80. At low temperature Gal4 is inhibited and expression of UAS-*p53* is repressed. Shifting larvae to 29 °C allowed expression of UAS-*p53* (see Materials and methods). Strikingly, when expressed in posterior cells early in the third instar, UAS-*p53* cell-autonomously induced processes characteristic of regeneration. Ectopic Wg expression was induced and compensatory proliferation occurred in posterior cells, resulting in overgrowth of the posterior compartment (Fig. 1D–D'). The overgrowth arose during a period of delayed larval development, suggesting that the developmental timer was affected (Fig. 1E). Apoptosis was also induced, as Caspase-3 (C3) was activated in the *p53*-expressing cells (Fig. 1D').

Interestingly, although the presence of the caspase inhibitor P35 exacerbated the responses to conditional *p53* expression, it was not required for them to occur (Fig. 1D–E). Taken together, these results provide evidence that p53 is necessary in a cell-autonomous manner after RH damage, and suggest that its expression during the third instar in wing discs is sufficient to induce the regeneration response.

The tissue repair response after IR requires p53

To test the generality of the p53 requirement during regeneration, we examined wing discs after induction of tissue damage by IR. We treated *yw* (used as a wildtype strain) and *p53* mutant larvae with 40 gray (Gy) of IR, a level sufficient to induce apoptosis in more than 50% of wing disc cells (Haynie and Bryant, 1976; Haynie and Bryant, 1977; Wichmann et al., 2006), at 96 h after egg laying (AEL). The p53 activity reporter was induced in the wing disc throughout the wing pouch within 3 h of IR in a p53-dependent manner, demonstrating that p53 was activated (Supp. Fig. 1A–D). After IR, p53 induces expression of Rpr and Hid, among other pro-death genes (Brodsky et al., 2004), thus to initially reproduce the conditions of RH damage, we expressed UAS-*P35* in posterior cells under control of EnGal4 (Wells et al., 2006). We then examined C3 activation to monitor apoptosis, ectopic Wg expression as a marker of regeneration, and disc overgrowth as a proxy for the regenerative compensatory proliferation (Wells et al., 2006).

In anterior wing disc cells, which did not express P35, C3 activation rapidly spiked after IR and then declined (McNamee and Brodsky, 2009), except for some cells near the anterior–posterior compartment boundary in which C3 staining persisted (Supp. Fig. 1E" and data not shown). Posterior cells, which expressed P35, contained high levels of C3 that persisted for at least 72 h after IR (Supp. Fig. 1E"). Patches of posterior cells expressed Wg ectopically, and the posterior compartment grew large and distorted (Supp. Fig. 1E'), indicating that the tissue repair program was induced. In contrast, evidence of this program was completely absent from wing discs of *p53* mutants after IR (Supp. Fig. 1F'–F" and data not shown). Thus, the IR-induced tissue repair response is p53-dependent, as it is after RH damage.

In RH-damaged tissue, a non-apoptotic function of the apical caspase Dronc is required to activate p53 (Wells et al., 2006). To determine whether Dronc was required to activate p53 in IR-induced regeneration, we examined wing discs carrying a *dronc* null mutation for evidence of regeneration. Loss of *dronc* prevents C3 activation and cell death in response to IR (Kondo et al., 2006). Interestingly, however, it did not prevent the tissue repair response after IR damage (Supp. Fig. 1G and data not shown). This result indicates that Dronc is not required to activate p53 after IR. In contrast, loss of *chk2* substantially suppressed the IR-induced response, consistent with its role as a DDR-induced p53 activator (data not shown) (Wichmann et al., 2006). Accordingly, disc regeneration after IR requires DNA checkpoint-dependent, but Dronc-independent activation of p53, suggesting that p53's function in disc regeneration is proximate to and contingent upon its activation in the DDR. Moreover, the finding that ectopic Wg, compensatory proliferation, and developmental delay (Supp. Fig. 1G and data not shown) occurred in IR-treated, *dronc* mutant discs demonstrates that cell death is not required to induce the tissue repair response.

Differentiation of adult structures after IR is compromised in the absence of p53

To follow the IR response of the disc in real time we did not express the caspase inhibitor P35 in our subsequent experiments. Larvae were irradiated and examined for developmental timing and for the ability of the wing discs to regenerate. IR at 96 h AEL induced a significant developmental delay of *yw* larvae such that pupariation occurred 27 h later than unirradiated controls (Fig. 2A). By contrast, irradiated *p53* mutant larvae did not significantly delay pupariation compared to unirradiated controls (Fig. 2A), similar to our findings of experiments with RH-induced damage (Wells et al., 2006). These results indicate that the systemic larval delay associated with both types of damage requires p53 function, and suggest that control of developmental timing after disc damage may be generally regulated by p53.

To determine how IR affected the formation of adults we examined the morphology and eclosion rates of adult flies that had been IR-treated at 96 h AEL. The vast majority of IR-treated *yw* animals differentiated appropriately and eclosed (Table 1). Of these, only 19% had minor morphological abnormalities, such as occasional missing thoracic machrochaetae (Table 2). As a proxy for regenerative growth we measured the size of the adult wings and found that after IR at 96 h AEL, wings of *yw* control flies grew to a similar size as unirradiated controls, indicating that regeneration had occurred successfully (Fig. 2D).

In striking contrast, although all IR-treated *p53* mutant animals pupariated and 78% of pupae differentiated and secreted adult cuticle, only 11% of these animals eclosed (Table 1 and data not shown). Eclosed *p53* mutant adults exhibited little movement and were unbalanced, shaky, and uncoordinated, suggesting damaged sensory and/or motor systems, and typically survived less than one day after eclosion (Supp. Fig. 2A). Moreover, IR resulted in numerous external defects of *p53* mutant adults, including notched and blistered wings, missing thoracic macro- and microchaetae, rough eyes and leg abnormalities (Table 2, Fig. 2C). In addition, wings from IR-treated *p53* mutants were 12% smaller than unirradiated *p53* mutants, a significant reduction in size (Fig. 2C–D). The range of morphological defects in uneclosed, irradiated *p53* mutant pharate adults extracted from the pupal case was similar to that of eclosed adults, suggesting that successful eclosion was not simply due to less severe damage (Table 2). Thus in the absence of p53, regeneration was significantly impaired.

Resolution of DNA repair following IR requires p53

IR-induced DNA damage is rapidly recognized by disc cells and the DDR is initiated, leading to a DNA repair program and apoptosis mediated by p53 (Brodsky et al., 2000; Rong et al., 2002; Sogame et al., 2003). The morphological defects in irradiated *p53* mutant adults prompted us to carry out a detailed examination of the DDR response in wing discs of *p53* mutant and control larvae. To this end we monitored the recognition of DNA damage, its repair, and apoptosis of IR-damaged cells in wing discs from *yw* controls and *p53* mutants at short intervals after IR. Phosphorylation of H2AX (γ-H2AX) is an early event in the recognition of DNA damage and antibodies against the mammalian form recognize γ-H2Av, its functional equivalent in *Drosophila* (Madigan et al., 2002), which was highly elevated in *yw* discs immediately after IR (Fig. 3H). γ-H2AX staining persisted in the *yw* disc cells for approximately 18 h but was cleared from the discs by 24 h after IR (Fig. 3B–B", H). Activated C3 increased and

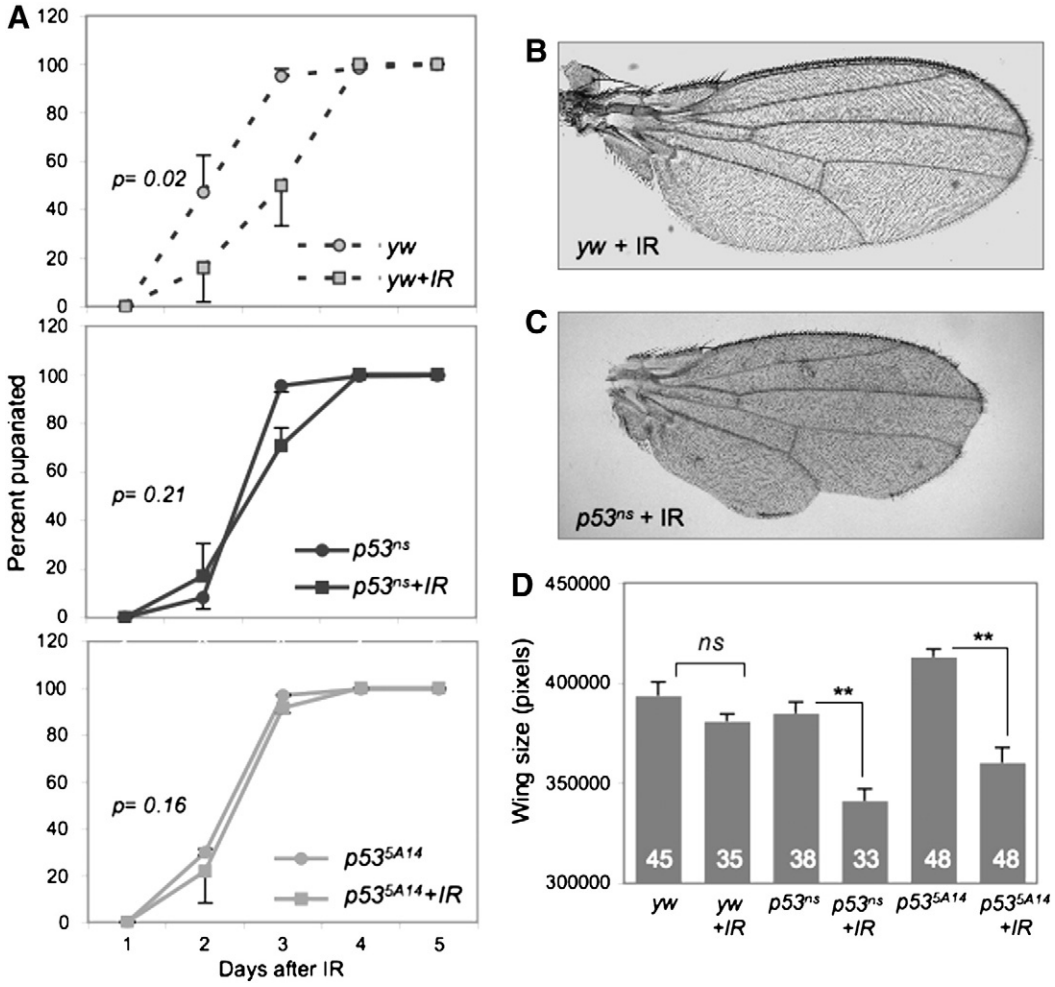

Fig. 2. IR induces a *p53*-dependent delay in developmental timing and leads to morphological defects. A. Irradiated *yw* larvae are significantly delayed relative to unirradiated controls. In contrast, irradiated $p53^{ns}$ or $p53^{5A14}$ mutants do not delay relative to unirradiated mutants. Animals were irradiated with 40 Gy at 96 h AEL. P values represent significance between irradiated and un-irradiated animals when 50% had pupariated. Error bars represent standard error of the mean from 6 independent experiments of at least 200 larvae per genotype. B–C. Representative wings from adult yw or $p53^{ns}$ animals irradiated at 96 h AEL. *p53* mutant wings are small and morphologically defective. D. Adult wing size of irradiated and unirradiated *yw* and $p53^{ns}$ and $p53^{5A14}$mutants. Wings of unirradiated and irradiated *yw* controls are similar in size, but wings of irradiated *p53* mutants were significantly smaller. Error bars denote standard error of the mean; n values for each genotype are listed. Larvae were irradiated with 40 Gy at 96 h AEL.

declined with similar kinetics (data not shown). Likewise, a steep increase in TUNEL-positive cells immediately followed IR, however in contrast to activated C3 and γ-H2AX, TUNEL-positive cells remained high for at least 48 h after IR (Fig. 3C–C", I). In addition, cell division arrested in control wing disc cells within 1 h of IR and remained arrested for approximately 5 h (Fig. 3A–A", G).

Cells in *p53* mutant wing discs also transiently arrested cell division after IR with similar kinetics as *yw* control disc cells (Fig. 3D–D", G) (Brodsky et al., 2000; Rong et al., 2002; Sogame et al., 2003). In addition, γ-H2AX staining was upregulated within minutes of IR in *p53* mutant wing discs (Fig. 3E–E", H), demonstrating that the recognition of DNA damage does not require p53. In contrast to controls, however, γ-H2AX staining persisted in 100% of wing discs of *p53* mutants, even far into the pupal stage (Fig. 3E–E", H; Fig. 6E", G"). This result differs from a report citing that γ-H2Av declined within a few hours in *p53* mutants, as in irradiated *yw* discs (see Supp. Fig. 3) (McNamee and Brodsky, 2009). Antibodies made against γ-H2Av recognized a subset of cells recognized by γ-H2AX antibodies (Supp. Fig. 3). The presence of γ-H2AX on DNA reflects ongoing DNA repair, and completion of repair leads to its dephosphorylation (Hromas et al., 2008; MacPhail et al., 2003; Olive, 2004), thus the persistence of γ-H2AX in the *p53* mutant disc cells suggests that p53 is required to resolve IR-induced DNA damage. These results are consistent with previous findings that p53 regulates expression of DNA repair genes and its loss leads to aneuploidy after IR (Brodsky et al., 2004; McNamee and Brodsky, 2009; Titen and Golic, 2008).

C3 activation and TUNEL-positive cells were largely absent from *p53* mutant wing discs up to 18 h following IR (Fig. 3F', I and data not shown), consistent with the requirement for p53 in death induced by the DDR (Brodsky et al., 2000; Rong et al., 2002; Sogame et al., 2003). Subsequently TUNEL positive cells increased dramatically and persisted into pupal development (Fig. 3F", I; Fig. 6C). This late

Table 1
Irradiated *p53* mutants have significantly reduced survival rates. Both irradiated (40 Gy at 96 h AEL) and unirradiated *yw* controls and *p53* mutant animals were assayed for differentiation during pupal stages (determined by cuticle deposition), as well as successful eclosion from pupal cases. Unirradiated larvae differentiate and eclose with nearly a 100% success rate and irradiated *yw* larvae differentiate and eclose with a very high level of success. Percentages of differentiating *p53* mutants are lower following IR (78%) and irradiated mutants are highly deficient in eclosion (11%). Values represent percentage ± standard deviation, number of animals scored are in parentheses.

	yw (1086)	*yw* + IR (572)	*p53* (952)	*p53* + IR (1679)
% Differentiated	99 ± 1	99 ± 1	99 ± 1	78 ± 5
% Eclosed	99 ± 1	83 ± 3	99 ± 1	11 ± 7

Table 2
p53 mutant adults exhibit numerous morphological defects following IR. Animals were assayed for gross morphological defects. Unirradiated animals exhibited no defects and irradiated *yw* animals had a low percentage of defects. In contrast, both eclosed and uneclosed (pharate) $p53^{ns}$ mutant adults exhibited similar and significant defects after irradiation (AIR). Values represent percentage ± standard deviation (when available); number of animals scored is in parentheses.

Phenotype (n)	Eclosed *yw* (69)	Eclosed *yw* + IR (51)	Eclosed *p53* (72)	Eclosed *p53* + IR (69)	Pharate *p53* + IR (24)
Notched wings	0	3 ± 1	0	45 ± 14	21
Blistered wings	0	1 ± 0	0	13 ± 0	nd
Missing thoracic bristles	0	19 ± 3	0	54 ± 13	63
Rough eyes	0	0	0	100 ± 0	100
Leg abnormalities	0	1 ± 1	0	29 ± 14	29

cell death corroborates previous reports of p53-independent late apoptosis after IR (McNamee and Brodsky, 2009; Wichmann et al., 2006), which may be activated in response to aneuploidy (Titen and Golic, 2008). Interestingly, both C3-positive and TUNEL-positive cells in irradiated *yw* wing discs were patterned and accumulated in particular around the boundaries of developmental compartments, suggesting input from the patterning system (Fig. 3C'–C") (Adachi-Yamada et al., 1999). By contrast, the late appearance of TUNEL positive cells in irradiated *p53* mutant discs was comparatively unpatterned (Fig. 3F"). We also observed TUNEL-positive cells with no obvious pattern in late larval *p53* mutant wing discs containing RH-induced tissue damage (data not shown). As JNK signaling is induced by and requires p53 after IR (data not shown) (McEwen and Peifer, 2005), and is also activated by distortions in patterning organizing activity (Adachi-Yamada et al., 1999), these observations indicate that p53 activity couples patterning information to JNK activation and apoptosis in IR damaged cells.

Growth of p53 mutant wing discs is not impaired by IR

The major proliferative phase of imaginal disc development occurs during the larval stage and is prolonged during tissue repair (Fig. 2A). The length of larval delay after disc injury generally correlates with the extent of damage, implying that it is related to the amount of growth needed to regenerate lost tissue (Poodry and Woods, 1990; Schubiger and Palka, 1987; Simpson et al., 1980). Our findings that irradiated *p53* mutants did not delay larval development and were also defective in aspects of the DDR suggested the possibility that disc growth was impaired in the mutants. To determine if this was the case, we followed the growth of wing discs by measuring their size at regular intervals after IR, from 96 h AEL until just prior to pupariation.

We first followed growth in our wildtype control strain. During the first 12 h after IR, wing discs from *yw* larvae grew at a similar rate as unirradiated controls. Subsequently, the rate of increase in disc size slowed significantly and remained slow during the extended larval

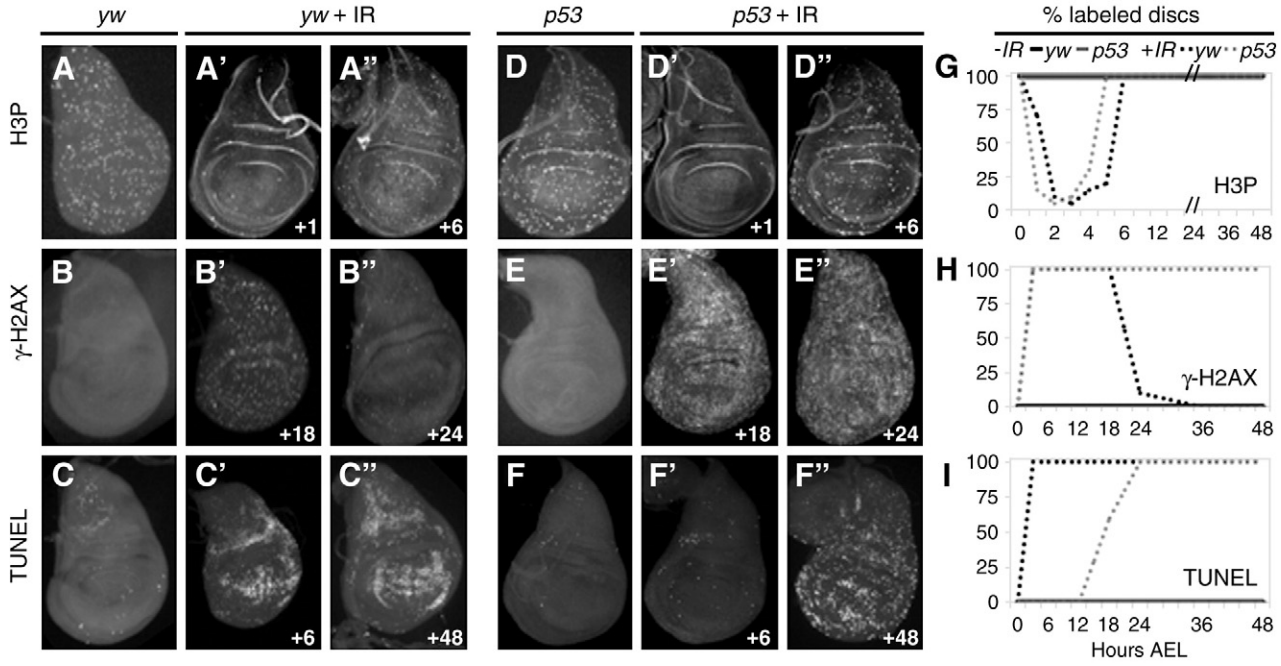

Fig. 3. DNA damage persists and apoptosis is delayed in irradiated *p53* mutants. A–A", D–D", G. IR-induced transient cell cycle arrest. discs from *yw* larvae (A–A") and $p53^{ns}$ larvae (D–D") show decreased H3P labeling at 1 h AIR, which returns to normal by 6 h AIR. A and D. Unirradiated *yw* and $p53^{ns}$ disc, respectively, at 97 h AEL. G. Both *yw* and *p53* wing discs show transient cell cycle arrest between 0 and 6 h AIR (dashed lines). Unirradiated *yw* and *p53* discs do not arrest (solid lines). B–B", E–E", H. DNA damage persists in *p53* mutant wing discs. B'–B". Irradiated control animals exhibit elevated levels of γ-H2AX from the onset of damage until 18 h AIR (B'). γ-H2AX is largely absent in control discs 24 h AIR (B"). B. Control disc—No IR, 114 h AEL. E'–E". γ-H2AX is elevated in *p53* mutant discs at 18 h AIR (E') and still at 24 h AIR (E"). E. Unirradiated *p53* mutant disc at 114 h AEL. H. γ-H2AX appears immediately after irradiation in *yw* and *p53* mutant discs. γ-H2AX disappears from *yw* discs after 18 h, but persist in $p53^{ns}$ wing discs. γ-H2AX was not detected in discs from unirradiated animals. C–C", F–F", I. *p53* mutants delay apoptosis AIR. C. *yw* disc—No IR, 102 h AEL. C'–C". *yw* animals upregulate TUNEL by 6 h AIR (C'), which persists more than 48 h AIR (C"). F. Unirradiated *p53* mutant disc at 102 h AEL. F'–F". *p53* wing discs do not label with TUNEL at 6 h AIR (F') but TUNEL increases later and is maintained until after 48 h AIR (F"). I. TUNEL labeling in *yw* animals AIR persists until pupariation. TUNEL is absent from *p53* mutant discs until approximately 18 h AIR. G–I. Graphs depict percent of discs expressing markers at indicated time AIR. Black lines denote *yw* animals, gray lines denote *p53* mutants, solid lines are unirradiated, dashed lines are irradiated. Animals were given 40 Gy IR at 96 h AEL. Time of dissection of irradiated animals is shown as hours following IR (bottom right of each panel). discs were dissected at 30-minute intervals until after 5 h; subsequently 6-hour time points were taken.

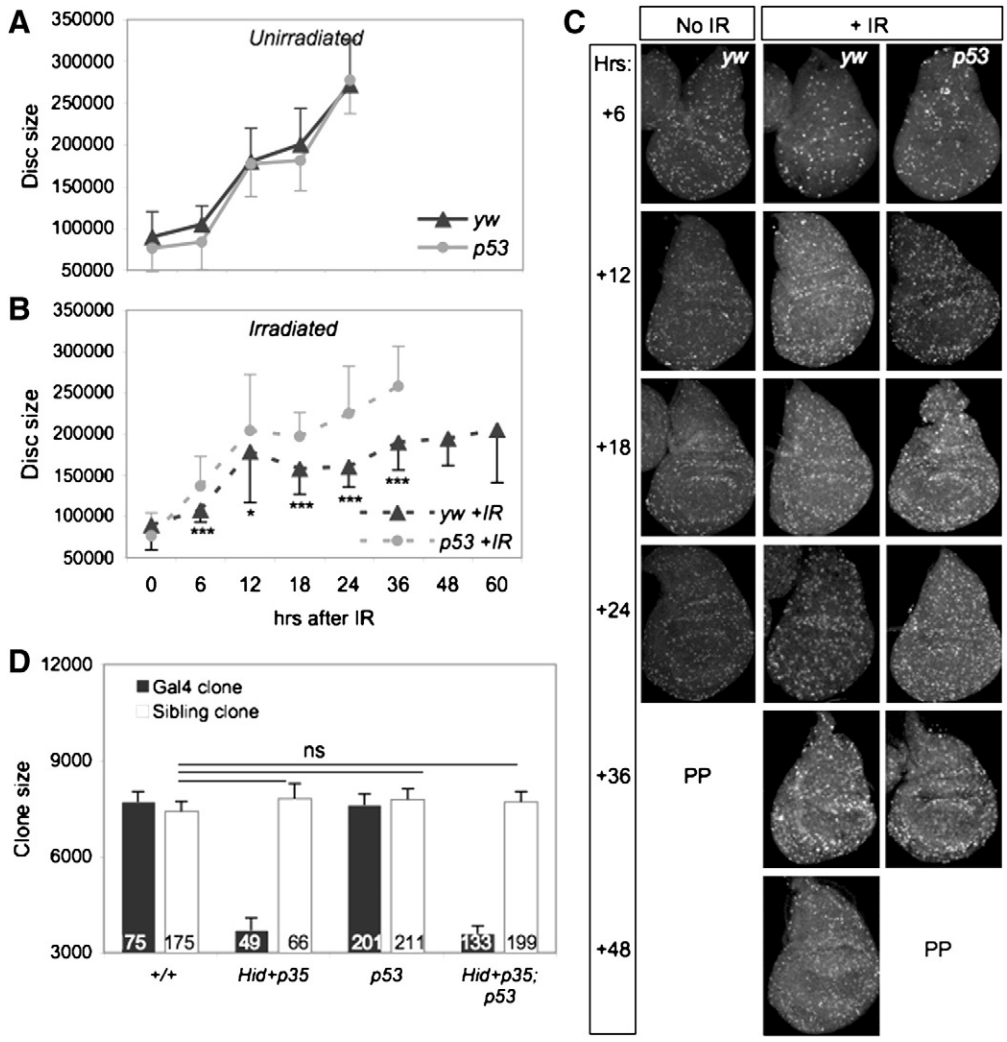

Fig. 4. Wing disc cell proliferation progresses at a developmentally programmed rate after IR and RH damage. A–B. Wing disc growth slows AIR. Animals were irradiated with 40 Gy at 96 h AEL (= 0 h). Black lines denote *yw* animals, gray lines denote $p53^{ns}$ mutants; solid lines are unirradiated, dashed lines are irradiated. A. Unirradiated *yw* and $p53^{ns}$ wing discs reach final size approximately 24 h AIR (120 h AEL). B. Wing discs from irradiated controls fail to reach final size by 50% PP (60 h AIR,156 h AEL) while wing discs of damaged $p53^{ns}$ animals reach final size by 50% PP (36 h AIR, 132 h AEL). 100% of larvae had wandered at the last point graphed for each genotype. $p53^{ns}$ mutant discs grew significantly more than *yw* discs at every timepoint indicated (* = <0.05, *** = < 0.001). Error bars denote standard deviation. C. Panels across each row show H3P-positive cells at 6-hour intervals after IR; intervals are noted as + hours post IR. At +6 h both irradiated *yw* and $p53^{ns}$ disc cells re-enter the cell cycle after the IR-induced arrest (see Fig. 3). H3P staining is distributed similarly in the discs regardless of genotype or irradiation. PP indicates entry into pupariation. D. MARCM experiment to measure cell proliferation in Gal4 clones with RH damage and their siblings. Black bars, Gal4 clones that express UAS-*GFP* alone (+/+ or $p53^{ns}$), and Gal4 clones that express UAS-*Hid* + UAS-*P35* and are either +/+ or *p53* mutant. White bars, wildtype (+/+) sibling clones growing next to RH expressing clones. All of the sibling clones grow at the same rate, indicating that proximate dying cells do not alter their rate of proliferation. Numbers of clones examined are indicated in each bar. Ns, not significant.

period (Fig. 4A–B). The slow growth of the discs occurred at the same time as the massive and ongoing apoptosis during this time (Fig. 3C'–C", I). Between 48 and 60 h after IR all of the irradiated *yw* larvae reached the wandering stage, yet their wing discs remained significantly smaller than those of unirradiated *yw* controls, which had wandered almost a day earlier (Fig. 2A, Fig. 4A–B).

By contrast, growth of *p53* mutant discs followed a different trend. In two independent experiments, *p53* mutant wing discs increased in size during the first 12 h after IR, slightly outpacing unirradiated controls in rate. After this period the growth of the mutant discs slowed; however, they reached the same size as unirradiated controls by late wandering stage (Fig. 4A–B). The slowing of disc growth coincided with the increase in late cell death in the *p53* mutants (Fig. 3F"), suggesting that an abrupt loss of cells led to a reduction in disc mass. Nevertheless, the average size of *p53* mutant discs was significantly larger than IR-treated *yw* discs at every timepoint examined after IR, even at the point when the larvae had entered wandering stage (Fig. 4B). Thus, *p53* mutant wing discs in irradiated larvae reached a normal size during larval development in the absence of additional time, suggesting that disc cells continue to proliferate at a relatively normal pace even after the onset of late apoptosis. We conclude that the small adult wing size of irradiated *p53* mutant larvae is not due to impaired growth during the larval stage.

Cell proliferation progresses at a developmentally programmed rate during regeneration

We then examined cell proliferation at 6 or 12 h intervals in the regenerating discs. After the resolution of the DDR-induced cell cycle arrest, mitotic, H3P-positive cells were similar in number and distribution in wing discs in both *yw* and *p53* mutants, and also similar to unirradiated discs (Fig. 4C, no IR versus + IR). This pattern in those discs continued with little variation for the next 36–48 h, although IR-treated discs continued to proliferate longer than unirradiated discs during the extended larval phase. H3P-positive cells became sparse as the larvae from both genotypes neared the pupariation stage, in keeping

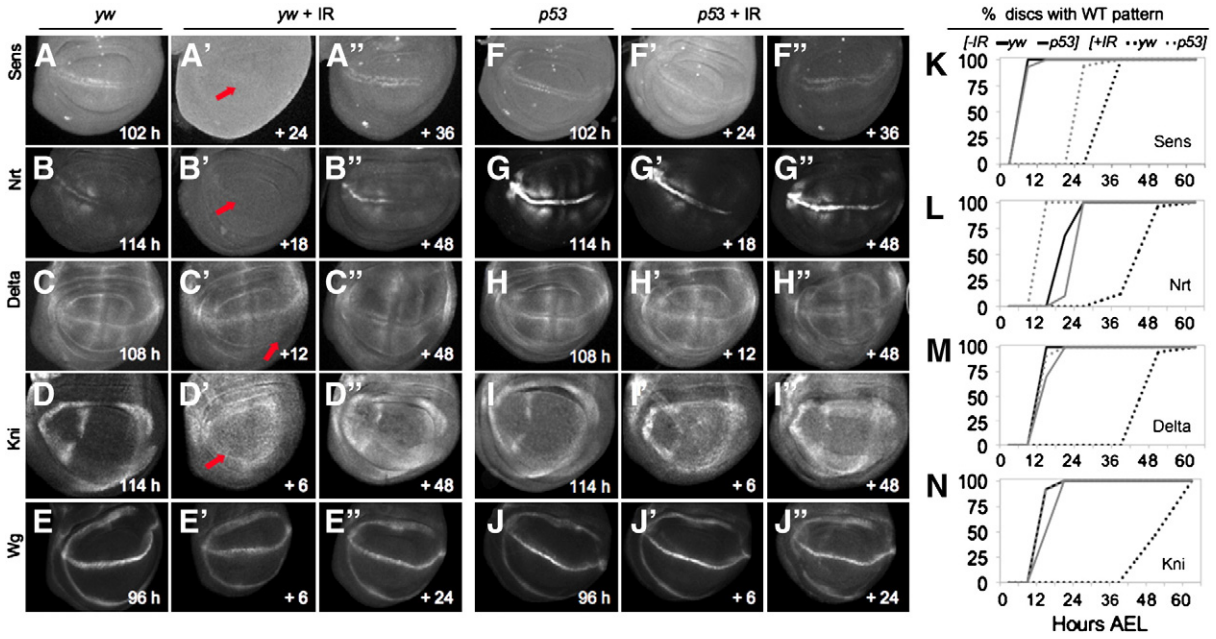

Fig. 5. A *p53*-dependent wing patterning checkpoint occurs after IR. A–J. Wing discs expressing the indicated patterning genes. Panels A–E and F–J represent the time when the mature pattern of each gene is observed in unirradiated *yw* and $p53^{ns}$ mutant discs, respectively. The age of unirradiated animals is shown in the bottom right of panels; for irradiated discs time is represented as hours after IR. A–A", F–F", K. Sens expression is delayed by IR. A. Sens expression at 102 h AEL in unirradiated discs. Following IR, Sens is absent (arrow) at 120 h AEL (A') and expression is delayed until ~132 h AEL (A"). F. Unirradiated *p53* mutants also express Sens at 102 h AEL. Following IR, *p53* mutants already express Sens at 120 h AEL (F'); the pattern is still present at 132 h AEL (F"). K. Quantification of mature Sens expression pattern in each genotype. B–B", G–G", L. Nrt protein expression is delayed by IR. B. Nrt expression at 114 h AEL in unirradiated discs. Following IR, Nrt is absent (arrow) at 114 h AEL (B') and expression is delayed until 144 h AEL (B"). G. Unirradiated *p53* mutants express Nrt at 114 h AEL, however AIR, *p53* mutants do not delay Nrt expression (G'–G"). L. Quantification of mature Nrt pattern in each genotype. C–C", H–H", M. Delta expression is delayed by IR. C. Delta is expressed at 108 h AEL in unirradiated discs. Following IR, Delta is absent (arrow) at 108 h AEL (C') and expression is delayed until 144 h AEL (C"). H. Unirradiated *p53* mutants express Delta at 108 h AEL. Following IR, *p53* mutants express the mature pattern of Delta by 108 h, which continues past 144 h AEL (H'–H"). M. Quantification of mature Delta pattern in each genotype. D–D", I–I", N. Kni expression is delayed by IR. D. Kni expression at 114 h AEL in unirradiated discs. Following IR, Kni is absent (arrow) at 120 h AEL (D') and expression is delayed until 144 h AEL (D"). I. Unirradiated *p53* mutants also express Kni at 114 h AEL. Following IR, *p53* mutants do not delay Kni expression (I') and expression continues past 144 h AEL (I"). N. Quantification of mature Kni pattern in each genotype. E–E", J–J". Wg is expressed in wing discs from the 2nd instar on and is mature by 96 h AEL in both unirradiated *yw* and *p53* mutants (E, J). Irradiating either *yw* or *p53* mutants does not grossly alter Wg expression (E'–E", J'–J"). K–N. Graphical representation of gene expression. Graphs depict the percent of discs with the mature pattern of expression of each gene at 6-hour intervals following IR. Black lines denote *yw* control animals, gray lines denote $p53^{ns}$ mutants; solid lines are unirradiated, dashed lines are irradiated. IR was induced with 40 Gy at 96 h AEL and time points (bottom scale) are taken from the time of IR (0 point = 96 h AEL).

with the temporary arrest of cell division near the larval-pupal transition (Schubiger and Palka, 1987). These results suggest that cell division during regeneration occurs at a developmentally programmed rate that is not altered by IR. Moreover, they indicate that the persistence of DNA damage in *p53* mutant disc cells did not interfere with their ability to proliferate.

To directly measure cell proliferation of the damaged cells, as well as non-damaged cells in their vicinity during regeneration, we generated RH-damage in wing discs via the MARCM system of mitotic recombination. This method avoids the stochastic nature of IR damage, and provides a powerful method of measuring compensatory cell proliferation stimulated by cellular loss or damage. We expressed UAS-*Hid* and UAS-*P35* specifically in marked Gal4 clones of cells and measured the growth of the Gal4 clones and their independently marked sibling clones after 40 h of growth (de la Cova et al., 2004). Control Gal4 and *p53* mutant Gal4 clones (Fig. 4D, black bars) and their siblings (white bars) grew at the same rate, and Gal4 clones expressing Hid + P35 grew poorly regardless of their genetic background (Fig. 4D, +/+ vs *p53*), as expected. Strikingly, sibling clones of either +/+ or *p53* mutant Gal4, UAS-*Hid* UAS- *P35* -expressing clones grew at the same rate as control sibling clones in discs with no RH damage (Fig. 4D).

All together, these results demonstrate that disc cell division progresses at a developmentally programmed rate during regeneration that is independent of p53 status, does not require repair of DNA damage, does not require cell death for its stimulation, and occurs at the same rate regardless of a developmental delay.

A p53-dependent patterning checkpoint in wing discs after IR

Our experiments indicate that IR induces two p53-dependent processes that contribute to disc regeneration: rapid induction of massive apoptosis, and slowed progression of the developmental clock to delay PP, allowing cell division – at a developmentally programmed pace – to replace lost cells. Since disc pattern formation and disc growth (increase in disc size) are tightly coupled, a delay in patterning might be expected in wing discs from IR-treated larvae. We therefore tracked the patterning process during regeneration by examining the expression of several patterning genes in wing discs from larvae treated with IR. Wg expression undergoes dynamic changes as development proceeds and is expressed ectopically during regeneration after concentrated tissue damage (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004; Wells et al., 2006). At 96 h AEL, the time of IR treatment, wing discs have a mature pattern of Wg expression: two rings encircling the central region with a bisecting line across the middle (Fig. 5E). We examined Wg expression at several timepoints after IR, and found no evidence of ectopic expression in controls or in *p53* mutants (Fig. 5E, J). This contrasts with the widespread, p53-dependent ectopic Wg seen after RH damage (Fig. 1B–C) (Huh et al., 2004; Ryoo et al., 2004; Smith-Bolton et al., 2009; Wells et al., 2006), or IR-induced damage when cell death is prevented by P35 or the *dronc* mutation (Supp. Fig. 1E–G) (Perez-Garijo et al., 2004). We attribute this difference to the stochastic and transient nature of IR damage. In addition, we did not detect a major loss of the mature pattern of Wg expression, suggesting that the cells maintained Wg expression in an

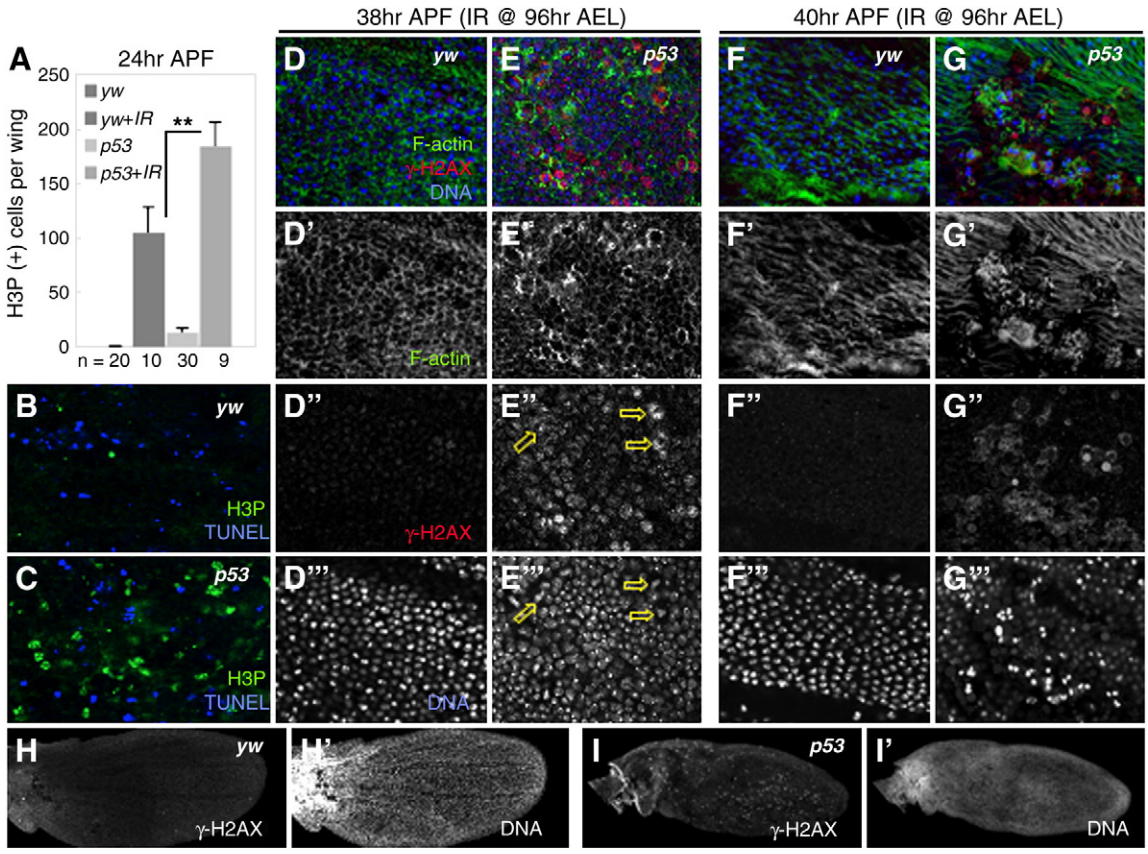

Fig. 6. Damaged cells persist into pupal development in *p53* mutants. A. Pupal wings have additional rounds of division that normally cease by 24 h APF. Pupal wings of irradiated *yw* and *p53* mutant animals continue to divide after this 24 h period. Irradiated *p53* mutants contain twice as many dividing cells as irradiated controls (**$p = 0.02$). B–C. At 24 h APF, pupal wings from irradiated *yw* controls and *p53* mutants have elevated levels of H3P and TUNEL. B. *yw*. C. *p53* mutant. D–D'''. Irradiated pupal wings from *yw* controls have cleared damaged cells by 38 h APF (D''), cells are hexagonal in shape and regularly packed (D', D'''). E–E'''. Irradiated *p53* mutant pupal wings at 38 h APF contain many γ-H2AX-positive cells, indicating that DNA damage persists (E'', arrows). DNA in many γ-H2AX-positive cells is aberrant (E''', arrows). Cells have become hexagonal in shape (E'), suggesting they are able to undergo some metamorphic processes but are frequently large and irregularly packed. F–F'''. By 40 h APF, pupal wing cells from irradiated *yw* animals secrete a pre-hair (F') and are free of damage (F''–F'''). G–G'''. Irradiated *p53* mutant pupal wings at 40 h APF begin forming pre-hairs (G'), but these remain absent in most γ-H2AX positive cells, which are still prevalent (G''). Many damaged cells appear to contain fragmented DNA or continue to attempt cell division (G'''). H–I'. At 38 h APF, pupal wings from irradiated *yw* animals have cleared nearly all damaged cells (H) and have begun vein formation (H'). *p53* mutant pupal wings still contain many damaged cells (I) and are much smaller and delayed in development relative to *yw* (I'; note lack of vein formation).

appropriate pattern after IR and did not revert to an earlier developmental stage.

We next addressed whether the onset of expression of patterning genes that initiate later in larval development was altered. A number of genes required for sensory organ precursor (SOP) and vein formation are initially expressed in mid-third instar wing discs, including *senseless* (*sens*), (Nolo et al., 2000, 2001), *Delta* (*Dl*) (O'Brochta and Bryant, 1987), *knirps* (*kni*) (Lunde et al., 1998) and *neurotactin* (*nrt*)

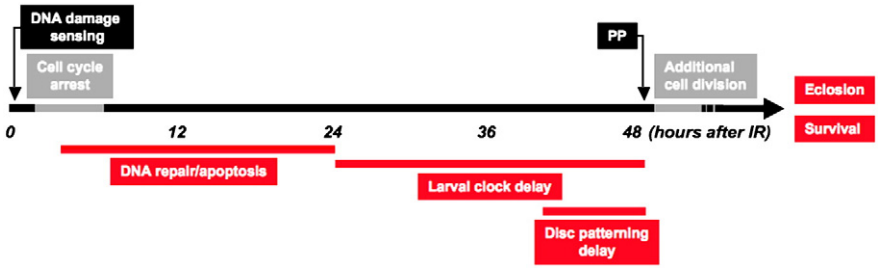

Fig. 7. Timeline of events induced after IR. IR interrupts the normal larval and imaginal disc developmental programs to induce a stereotypical cell and tissue repair response. The response initiates when DNA damage is sensed, a cell cycle arrest is enacted, followed by cell cycle re-entry. These initial processes of the response are *p53*-independent (time scale is approximate and reflects hours AIR). The onset of cell death is followed by a systemic delay that allows for DNA and tissue damage repair. Massive apoptosis occurs in wing discs, eliminating many cells, while after reentry into the cell cycle cell division continues at normal rates over a prolonged period of growth. disc cells delay expression of late patterning genes. DNA repair is essentially complete within 24 h AIR. disc patterning resumes despite small overall disc size, and the animals ultimately make the larval–pupal transition. Cell division continues longer than normal in pupal discs and discs reach appropriate final size. Animals eclose as healthy adults after an extended pupal period. p53 is required for many subsequent events to occur (indicated by red lines). *p53* mutants do not significantly delay development. DNA repair is unsuccessful and damaged DNA persists. Continued cell division at the normally programmed rate and a lack of apoptosis results in temporarily appropriate wing disc growth, but most late patterning occurs on the normal developmental schedule. 18 h AIR cells begin to die, probably due to aneuploidy. Despite this, larvae make the larval–pupal transition; expression of γ-H2AX persists in disc cells leading to defects in cell shape and size. disc cells continue to divide but many apoptose, reducing overall wing size; pupal development is extended for approximately 36 h beyond the norm. Animals differentiate with morphological defects. Few adults eclose, and viability is severely reduced.

(Speicher et al., 1998). Each of these genes has a dynamic expression pattern in the wing disc that either begins or changes after 96 h AEL, the time at which larvae were subjected to IR in our experiments. In unirradiated *yw* animals *sens* was expressed in SOP cells along the D/V boundary of wing discs at 102 h AEL (100% of discs; Fig. 5A, K). Similarly, *Dl* was expressed in the presumptive L5 vein region at 108 h AEL (100%; Fig. 5C, M), *kni* was expressed in the presumptive L2 vein region by 114 h AEL (100%; Fig. 5D, N), and *nrt* was expressed at the D/V boundary by 120 h AEL in 100% of unirradiated *yw* wing discs (Fig. 5B, L). Following IR of *yw* animals, expression of each of these genes was delayed: *sens* expression by 30 h (100% of discs); *Dl* (100%) by 48 h, *kni* (100%) by 42 h, and *nrt* (100%) by 32 h (Fig. 5A'–D', K–N). The delayed expression patterns of these genes correlated well with the delay in larval development after IR. However, despite the temporal delay, the spatial pattern of expression of each gene in irradiated animals was identical to unirradiated controls.

In unirradiated *p53* mutants, these genes were expressed at similar times: *sens* by 108 h (100% of discs), *Dl* by 114 h (100%), *kni* by 114 h (100%) and *nrt* by 120 h AEL (100%; (Fig. 5F–I, K–N). However, in striking contrast to *yw*, in IR-treated *p53* mutants most of the genes were expressed with normal timing: *Dl* (100%) and *kni* (100%) expression occurred with normal timing, and *nrt* expression was induced at 108 h AEL (100% of discs), prior to its onset in unirradiated *yw* animals (Fig. 5G–I, L–N). Only *sens* expression was delayed in the mutants, although the delay was reduced relative to irradiated *yw* controls (Fig. 5F–K). Thus, in wildtype animals the IR-induced delay of the larval stage is accompanied by a delay in the onset of expression of several late patterning genes in the wing disc, and both of these delays are p53-dependent.

Cell proliferation continues during pupal development after IR

Our finding that wandering stage wing discs from IR-treated *yw* larvae were smaller than comparably aged discs from unirradiated controls (Fig. 4A–B) suggested that the delay to PP was not sufficient to regenerate wing discs to an appropriate size. Nevertheless, adult wings arising from irradiated larvae were similar in size to unirradiated larvae, indicating that ultimately, regeneration was complete (Fig. 2B, D). These results suggested that IR-treated wing disc cells continue to divide beyond the time cell division normally ceases in pupal development. Supporting this idea, the pupal stage was extended by 29 h in IR-treated *yw* control larvae (Supp. Fig. 2C). Interestingly, irradiated *p53* mutants also extended the pupal stage (Supp. Fig. 2C), indicating that in contrast to the larval delay, the pupal extension was not p53-dependent. To determine its consequences on wing regeneration we examined pupal wing discs, developed from irradiated larvae, for markers of cell division, cell death and DNA damage. Cell proliferation normally ceases by 24 h after puparium formation (APF), thus few cells were positive for H3P in unirradiated wing discs from *yw* controls at this time. However, pupal discs from IR-treated *yw* larvae contained significant numbers of H3P positive cells at 24 h APF (Fig. 6A). Cell division continued for approximately 14 additional hours in these discs, finally ending by 38 h APF (data not shown). Coupled with our findings that regenerating *yw* larval wing discs were small at the larval–pupal transition but reached a normal adult size, these results suggest that the proliferation during the pupal stage was necessary to complete regeneration of the damaged tissue.

We then examined pupal wing discs from *p53* mutants. In the absence of IR, cells in *p53* mutant discs stopped dividing with normal kinetics, and few H3P positive cells were observed in wing discs 24 h APF (Fig. 6A). However, many cells in IR-treated *p53* mutant pupal wing discs were mitotic, and at 24 h APF we observed twice the number of H3P positive cells relative to IR-treated *yw* pupal discs ($p = 0.02$; Fig. 6A–C). Cell division in the *p53* mutant discs also continued longer than in *yw* discs: at 40 h APF, *p53* mutant pupal wings still contained numerous H3P positive cells (data not shown).

Unrepaired, damaged cells have limited differentiation potential

The additional cell proliferation in pupae helped to explain how wings of control flies irradiated during L3 reached an appropriate final size. In contrast, despite similar extensions of the pupal stage (Supp. Fig. 2C) and continued cell proliferation (Fig. 6A), *p53* mutant pupal wing discs (Fig. 6H–I) and adult wings (Fig. 2C–D) remained significantly smaller than controls. The persistent γ-H2AX in larval discs implied that DNA damage was not repaired, thus we examined pupal wing discs from IR-treated *yw* and *p53* mutant animals in more detail. At 24 h APF, pupal wing discs from *yw* and *p53* mutants were undersized relative to unirradiated controls, and discs of both genotypes had numerous apoptotic cells (Fig. 6B–C and data not shown). However, although the *yw* pupal discs contained numerous TUNEL positive cells, very few contained γ-H2AX positive cells at 24 h APF (data not shown) (Fig. 6B). Apoptosis subsided by 40 h APF and few cells in *yw* pupal wing discs were TUNEL positive at this time (data not shown).

In contrast, cells with DNA damage persisted in the *p53* mutant pupal discs. 24 h APF, *p53* mutant cells frequently contained γ-H2AX (data not shown). TUNEL-positive cells were also common in these discs but they rarely coincided with γ-H2AX-positive cells, suggesting that damaged cells were not always eliminated (Fig. 6C and data not shown). We also found that *p53* mutant cells were frequently substantially larger than *yw* cells of the same chronological age (Fig. 6E' vs D'), and some appeared to be aneuploid, suggesting aberrant mitoses had occurred (Fig. 6E'", arrows; Supp. Fig. 4C–C' and data not shown). Consistent with this idea, many cells contained both H3P and γ-H2AX (data not shown). At 38 and 40 h APF, *p53* mutants still maintained high levels of γ-H2AX and many TUNEL positive cells, but again these markers rarely overlapped (Fig. 6E", G" and data not shown). Collectively, these results indicate that the small pupal wing discs and adult wings of IR-treated *p53* mutants result from a combination of a late wave of apoptosis that eliminates many damaged cells, and continued, unproductive cell divisions due to persistent and unresolved DNA damage.

By 38 h APF, IR-treated wing disc cells from *yw* animals became hexagonal in shape (Fig. 6D) and each formed a pre-hair before 40 h APF (Fig. 6F'). These processes are associated with cell differentiation and are connected in time during wing morphogenesis (Classen et al., 2005; Waddington, 1941). However, in IR-treated discs they occurred approximately 10 h later than normal, consistent with the overall delay in the pupal stage. Interestingly, even γ-H2AX-positive cells in *p53* mutant pupal wings become hexagonal (Fig. 6E' and data not shown). By contrast, although non-damaged cells formed pre-hairs, most of the cells with DNA damage (γ-H2AX-positive) did not (Fig. 6G'), suggesting that differentiation of the cells was limited. Each pre-hair forms a trichome, which is displayed with proximal to distal polarity in a regularly-spaced pattern across the adult wing, and trichome density is an indicator of cell size prior to cuticle secretion (Dobzhansky, 1929). Regenerated wings from IR-treated *yw* animals were patterned with correct trichome polarity (Fig. 2B). Although the overall wing size of IR-treated *p53* mutants was reduced and wings frequently had defects (Fig. 2C–D; Table 2), trichome density and polarity of eclosed *p53* mutants was similar to regenerated *yw* controls (Supp. Fig. 2B). The trichome density indicates that the small wing size in the IR-treated *p53* mutants was due to fewer, rather than smaller cells. Together, these observations suggest that the IR-treated *p53* mutant cells had limited differentiation potential due to the persistence of damage, and that many damaged cells were ultimately eliminated from the epithelium.

Discussion

In this work, we make several key findings about imaginal disc regeneration. First, we find that both RH- and IR- induced tissue damage require p53 function for regeneration, but each process utilizes a different genetic mechanism to activate p53, suggesting that p53

plays a universal role in regulating regeneration. Second, we demonstrate that cell death is not necessary for regeneration to be induced, indicating that the lack of the early cell death program in *p53* mutant wing discs cannot explain their inability to regenerate. Third, we find that cell proliferation is not stimulated during regeneration, but progresses at a developmentally programmed pace. Fourth, we identify a developmental checkpoint that controls the timing of disc patterning during the regeneration process. Finally, our data indicate that compensatory proliferation is not restricted to the larval stage of development. We discuss these findings below.

p53 functionally coordinates the DDR with tissue regeneration

Our results show that tissue regeneration subsequent to the DDR also requires p53. They add to previous work indicating that a continuum of events follows IR that culminates in regeneration of damaged tissue and survival of the animal (Fig. 7). The process initiates with a stereotypical DDR in damaged imaginal disc cells within minutes of IR: damage is sensed and H2AX is phosphorylated, caspases are activated, and cell division in the disc transiently arrests. After approximately 5 h disc cells re-enter the cell cycle and continue to divide at apparently normal rates. Repair of DNA damage leads to loss of γ-H2AX, while ongoing apoptosis eliminates unrepaired cells. Our results indicate that the high level of cell death significantly slows the net growth of wing discs, compelling continuous cell division. This is facilitated by a delay of pupariation; in parallel, the expression of late patterning genes is delayed in wing discs. Interestingly, we found that after 40 Gy of IR, tissue damage was severe enough to require disc cell proliferation to continue not only during the extension of the larval developmental timer, but also into the pupal stage. Thus in contrast to what has been generally believed, disc regeneration is not restricted to the larval "growth phase" of development, but can continue in the early stages of pupal development. The ability of discs to continue regenerative growth after the hormonal cues that stimulate pupariation suggests that disc cell proliferation is only loosely regulated at the juvenile-adult transition.

We find that in *p53* mutants the events following IR are initially identical to wildtype, but subsequently show several differences. Cells lacking *p53* recognize DNA damage and H2AX is phosphorylated, and the cell cycle checkpoint transiently arrests *p53* mutant disc cells. *p53* mutant cells also reenter the cell cycle with the same kinetics as controls. However, γ-H2AX persists at high levels in mutant discs, indicating that DNA damage is lingering, but the cells are unable to undergo apoptosis. Moreover, the mutant larvae do not significantly delay development, suggesting that p53 is required to regulate the developmental timer. Despite these differences, we found that disc cells divide at a normal rate and thus the size of the wing disc initially increases after IR. Later, the persistence of damaged DNA coupled with cell division creates aneuploidy, which, as noted previously (Titen and Golic, 2008), may contribute to a late wave of apoptosis that we find continues late into the pupal stage.

Cell division continues beyond the normal cessation time in both our wildtype strain and the p53 mutants. In wildtype these additional pupal cell divisions are productive. In contrast, the late pupal divisions of *p53* mutant disc cells appear to be largely futile since cell death is still prevalent; surviving cells appear to be aneuploid. Perhaps as a result, wing morphogenesis is delayed in irradiated *p53* mutants relative to controls, although the mechanisms that pack wing disc cells and re-shape the wing disc ultimately do occur. The lack of DNA repair impairs cell differentiation and/or function throughout the pupa and leads to defects that prevent most animals from eclosing. This is interesting in light of our finding that the persistent DNA damage in *p53* mutants did not appear to interfere with cell division during the larval phase of regeneration.

We found that p53 functions cell autonomously during disc regeneration, and that conditional expression of p53 in the wing disc is sufficient to induce ectopic expression of Wg, compensatory tissue growth, and a systemic developmental delay, all common aspects of regeneration. These results suggest that p53 is activated and operates cell-autonomously in damaged cells to promote regeneration. However, p53 also regulates the larval developmental clock, with the result that it coordinates control of disc regeneration with the physiology of the whole animal. Collectively, our results indicate that p53 functions to ensure repair of damaged DNA, to regulate the developmental timing of the animal, and to coordinate disc and animal maturation via a patterning checkpoint that delays cell fate acquisition in the disc. This linkage provides a mechanism that coordinates the two processes in time and thus facilitates the survival of the animal after DNA and tissue damage.

Repair of damaged DNA is critical for tissue regeneration

Our experiments show that in the absence of p53, DNA damage remains unrepaired, rendering cells incapable of completing the differentiation process. This is exacerbated by the absence of apoptosis immediately after IR in the *p53* mutants, allowing cells with DNA damage to persist. Later, some of the persisting damaged cells in the mutants are eliminated by a late surge of disc cell death that continues into the pupal stage, in accordance with previous reports (McNamee and Brodsky, 2009; Wichmann et al., 2006). However, although this rids the disc of many damaged cells, it is not induced within a time frame that allows replacement of lost tissue, leading to small pupal wing disc size and small adult wings. Shortly after the onset of the late wave of apoptosis in *p53* mutant discs the larval–pupal transition is crossed and metamorphosis is initiated. Although *p53* mutant pupal wing disc cells continue to proliferate long after their wildtype counterparts have exited the cell cycle, our results suggest that the juvenile-to-adult transition – the commitment to produce adult traits – prevents critical developmental and patterning cues or render cells incapable of responding to them. However, damaged, aneuploid cells can differentiate trichomes (Wichmann et al., 2006), and we observed that some damaged cells did carry out aspects of trichome differentiation, including prehair formation. In addition, it is possible that most of the severely damaged cells in p53 mutants were ultimately eliminated.

Our results are strikingly similar to observations made in mouse and human cells. Loss of the murine DNA damage checkpoint protein Hus1 in a *p53*-deficient background results in accumulation of damaged cells after IR and prevents the compensatory responses in mammary epithelium (Yazinski et al., 2009). In serial transplantation experiments, self-renewal of irradiated human hematopoietic stem cells (HSCs) is compromised when they are deficient for p53 (Milyavsky et al., 2010), and, like our experiments with wing disc cells, γ-H2AX persisted in the HSCs. Collectively the data indicate that p53's role in *Drosophila* disc regeneration is analogous to its role in tissue remodeling and stem cell renewal in vertebrates, and suggest that these functions of p53 are conserved.

p53 and systemic versus local effects during regeneration

The argument can be made that *Drosophila* imaginal discs merely take advantage of and extend developmental programs to repair and re-pattern lost tissue. This requires that the appropriate hormonal milieu be maintained by prolonging the juvenile, larval stage. We irradiated animals late during larval development but still within the disc growth period, and found that p53 function is required for the delay of the developmental timer that controls the juvenile-adult transition. Likewise, delay of the timer after RH-damage requires p53 (Wells et al., 2006). There is a strong correlation between delay of the timer and continued proliferation of discs (Bryant and Simpson, 1984; Cruz et al., 2009; Ramet et al., 2002). Although this relationship remains mysterious it is generally thought that negative feedback from proliferating discs

inhibits a neural or humoral target (Poodry and Woods, 1990). In contrast to imaginal discs, the polyploid larval cells are relatively insensitive to IR (Halme et al., 2010; Poodry and Woods, 1990). We were unable to detect induction of p53 activity after either RH damage or IR in tissues known to play key roles in developmental timing, such as the fat body, Dilp-2 expressing neurons, the prothoracic gland, and the corpora allota (data not shown). The p53 activity reporter contains 2 consensus p53 binding sites and is thus expected to report accurately in many tissues. Although we cannot exclude more trivial possibilities, the absence of induction in these tissues suggests that p53 function in imaginal discs is sufficient for the developmental delay induced upon IR as well as for the disc-autonomous responses. Thus, our data support the view that imaginal discs "signal" to the developmental clock to delay pupariation, and indicates that the putative signal requires p53 for its production.

Compensatory cell proliferation proceeds at a developmentally programmed rate during regeneration

We have presented two independent lines of evidence that argue that, in contrast to previous reports (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004), cell proliferation occurs at the same rate during regeneration as it does under normal developmental conditions. First, we found the number and distribution of mitotic cells to be similar in *yw* and in *p53* mutant discs following IR at every examination from the cell cycle reentry at +6 h until pupariation, despite the significant differences in the length of this period between the two genotypes. Second, RH-damage in clonal experiments showed that undamaged cells in the vicinity of RH-damaged cells proliferate at the same rate as cells in control discs without damage (Fig. 4D) (Wells et al., 2006). Our results agree with Haynie and Bryant (Haynie and Bryant, 1977), who observed an IR dose-dependent lengthening of the developmental timer that correlated with an increase in clone size in adult wings, and concluded that the remaining cells "undergo additional divisions to compensate for this loss". As a whole the data indicate that cell divisions occur at the normal rate, with additional divisions that occur during a p53-dependent slowing of the larval timer.

In addition, our results indicate that some aspects of disc patterning are delayed while the disc regenerates. This delay is also p53-dependent. One interpretation of these results is that the early, p53-dependent cell death program, by eliminating massive numbers of cells, directly delays the ongoing patterning process. However, our finding that *dronc* mutant animals, which are unable to induce cell death, exhibit the same regeneration responses as wildtype after IR argues against this idea. An alternative possibility arises from the observation that the disc patterning delay and the animal-wide delay are correlated in time, and thus could be inter-dependent. A third possibility is that p53 induces a disc-wide developmental checkpoint, directly dependent upon its role in the DDR but independent of the disc-produced "signal" that delays the larval timer, which couples regenerative growth to stage-appropriate cell fate specification. Further experiments are required to distinguish between these alternatives. Regardless of the mechanism, however, our finding that cell division proceeds at a similar rate during the delay of patterning regardless of p53 status implies that cell division and late patterning gene expression are independently regulated under these conditions.

What is the regeneration trigger?

It has been hypothesized that dying cells emit information that stimulates proliferation of surviving cells to regenerate the damaged tissue (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). The regulation of expression of pro-apoptotic genes and pathways such as Rpr, Hid, Eiger/TNF and JNK by p53 (Brodsky et al., 2004; McEwen and Peifer, 2005), is consistent with the idea that p53 induces apoptosis, which in turn stimulates regeneration. However, our results indicate that this is not the case: the regeneration response is induced after IR even in cells rendered incapable of inducing apoptosis because of a null mutation in *dronc*. Since these *dronc* mutant cells remain wildtype for p53, these data support an apoptosis-independent role of p53 in provoking regeneration. Indeed, p53 is required for the tissue repair response even when RH genes are expressed (Wells et al., 2006). Thus, at a minimum, the data indicate that expression of pro-death genes and caspase activation are not sufficient to trigger regeneration.

As regeneration does not occur in its absence, p53 appears to be upstream of the signal that triggers regeneration. We suggest three scenarios for the regeneration trigger downstream of p53 activation. First, we find that p53 functions cell-autonomously to promote the ectopic induction of Wg, an early event in regeneration induced by a variety of methods in numerous animals (De Robertis, 2010; Galliot and Chera, 2010; McClure and Schubiger, 2008; Ryoo et al., 2004; Smith-Bolton et al., 2009; Wells et al., 2006). Moreover, expression of p53 under conditional Gal4 control induces ectopic Wg in the wing disc. Thus, the re-organization of disc patterning of the damaged tissue by ectopic expression of Wg, which necessitates a developmental delay for the completion of patterning and growth, may serve to trigger regeneration. However, in contrast to previous models invoking Wg as a mitogen, our findings indicate that cell proliferation continues at its developmentally programmed pace during this extended period of time.

Since JNK activation after tissue damage is p53-dependent (our unpublished data) (McEwen and Peifer, 2005), a second candidate for the regeneration trigger is the JNK signaling pathway. JNK is activated early after tissue damage and is important for wound healing (Bergantinos et al., 2010b; de Celis et al., 1996a; de Celis et al., 1996b; Galko and Krasnow, 2004; Hay et al., 1995; Mattila et al., 2005). JNK signaling is also activated upon disruptions of Wg and Dpp in the wing disc (Adachi-Yamada et al., 1999), and can itself lead to activation of Wg and Dpp expression (Perez-Garijo et al., 2009). JNK activity appears to be upstream of Wg expression, since *hep* null mutations, which eliminate JNK activity, prevent ectopic expression of Wg after RH damage (our unpublished data). A third possibility is that regeneration is triggered via a distinct program of gene expression directed by p53, which is independent of JNK or Wg.

Overall, our work suggests that p53 acts as a master regulator of tissue plasticity through its roles in the DDR, in tissue repair, and in coordinating these events with the animal's physiology. In addition to its role in the initiation of regeneration, our results argue that p53 is responsible for regulating the expression of a signal(s) from discs that prolongs larval development to allow regeneration after either RH or IR damage. Studies that identify this signal, that determine from which tissue it arises, and that delineate the mechanism by which p53 controls each aspect of the regeneration process are important goals for the future.

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Appendix A. Supplementary data

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